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1) Tanner, 1994, Cancer Res, 54: 4257-4260
2) Gyapay, 1994, Nature Genetics, 7: 246-339. 1455-1461
3) TANNER M M , 1995, CLIN CANCER RES, 1(12): ~~1445~~-61

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Advances in Brief

Amplification of Chromosomal Region 20q13 in Invasive Breast Cancer: Prognostic Implications[1]

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Abstract

Amplification of the chromosome 20q13 region was recently discovered in breast cancer by comparative genomic hybridization and subsequently further defined by fluorescence *in situ* hybridization with specific probes. The target gene of the amplification remains unknown. Here, fluorescence *in situ* hybridization with a cosmid probe for the minimal region of amplification (RMC20C001) was used to study 20q13 amplification in 132 primary breast carcinomas and 11 metastases. The size of the amplicon was studied with four flanking probes. Thirty-eight (29%) primary tumors and 3 (27%) metastases showed increased copy number of the RMC20C001 probe (>1.5-fold relative to the p-arm control). Nine (6.8%) of the primary tumors were highly (>3-fold) amplified. Although the size and location of the amplified region varied from one tumor to another, only the RMC20C001 probe was consistently amplified. 20q13 amplification was significantly associated with a high histological grade ($P = 0.01$), DNA aneuploidy ($P = 0.01$), and high S-phase fraction ($P = 0.0085$). High-level amplification was also associated with short disease-free survival of patients with node-negative breast cancer ($P = 0.002$). We conclude that high-level 20q13 amplification may be an indicator of poor clinical outcome in node-negative breast cancer and that this chromosomal region is likely to contain a gene with an important role in breast cancer progression. A large definitive study is warranted to assess the independent prognostic value of 20q13 amplification.

Introduction

DNA amplification may lead to up-regulation of gene expression and represents an important mechanism for oncogene activation (1, 2). Several oncogenes, such as *erb-B2* (at 17q12), *myc* (8q24), and *cyclin D/EMS1* (11q13) are known to be amplified in 10–25% of primary breast carcinomas. These amplifications have often been reported to be associated with a poor prognosis for the patients (3–5).

Chromosomal region 20q13 was recently found to be amplified in breast cancer based on the genome-wide copy number analysis by CGH[3] (6). According to CGH, 20q13 amplification was found in 12–18% of selected primary breast tumors and 40% of cell lines (7, 8). Chromosomal microdissection shows a similar prevalence (44%) for 20q13 amplification in breast cancer cell lines (9) and allelic imbalance at 20q13 has been reported in 16% of primary tumors (10). Recently, we used FISH with region- and gene-specific probes to study overlapping amplicons in different breast tumors (11). The minimal common region of amplification was tentatively narrowed down to 1.5 megabases at 20q13.2, and all candidate genes were excluded. This region of interest lies in the vicinity of genetic markers *D20S120* and *D20S480*[4] and between genes *MC3R* (fractional length from pter 0.81) and *PCK* (0.84).

Since the target gene for this amplification remains unknown, the biological and clinical associations of 20q13 amplification could give insights to the function of this gene. In the present study, we used interphase FISH to study tumor nuclei from 132 primary breast cancers and 11 metastases. The aim was to determine the prevalence and significance of 20q13 amplification in breast cancer.

Materials and Methods

Patients and Tumor Material. Tumor samples were obtained from 152 women who underwent surgery for breast cancer between 1987 and 1992 at the Tampere University or City Hospitals. One hundred forty-two samples were from primary breast carcinomas and 11 from metastatic tumors. Specimens from both the primary tumor and a local metastasis were available from one patient. Ten of the primary tumors that were either *in situ* or mucinous carcinomas were excluded from the material, since the specimens were considered inadequate for FISH studies. Of the remaining 132 primary tumors, 128 were invasive ductal and 4 invasive lobular carcinomas. The age of the patients ranged from 29 to 92 (mean, 61) years. Clinical follow-up was available from 129 patients. The median follow-up period was 45 (range, 1.4–77) months. Radiation ther-

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[3] The abbreviations used are: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; DAPI, 4,6-diamidino-2-phenylindole; ER, estrogen receptor; PR, progesterone receptor.

[4] C. Collins, manuscript in preparation.

apy was given to 77 of the 129 patients (51 patients with positive and 26 with negative lymph nodes), and systemic adjuvant therapy was given to 36 patients (33 with endocrine and 3 with cytotoxic chemotherapy). Primary tumor size and axillary node involvement were determined according to the TNM classification. The histopathological diagnosis was evaluated according to the WHO (12). The carcinomas were graded on the basis of the tubular arrangement of cancer cells, nuclear atypia, and frequency of mitotic or hyperchromatic nuclear values according to Bloom and Richardson (13).

Surgical biopsy specimens were frozen at −70°C within 15 min of removal. Cryostat sections (5–6 μm) were prepared for intraoperative histopathological diagnosis, and additional thin sections were cut for immunohistochemical studies. One adjacent 200-μm-thick section was cut for DNA flow cytometric and FISH studies, and one 100-μm-thick section was cut for DNA isolation.

Cell Preparation for FISH. Tumor specimens were first histologically examined, and only those containing more than 60–70% malignant epithelial cells were considered representative for FISH analysis. Nuclei were isolated from 200-μm frozen sections according to a modified Vindelov procedure for DNA flow cytometry, fixed, and dropped on slides for FISH analysis as described (14). Foreskin fibroblasts were used as negative controls in amplification studies and were prepared by harvesting cells at confluency to obtain G_1-enriched interphase nuclei. All samples were fixed in methanol-acetic acid (3:1).

Probes. Five probes previously mapped to the 20q13 region were used in this study (15). The probes included PI clones for melanocortin-3 receptor [probe MC3R, fractional length from p-arm telomere (Flpter) 0.81] and phosphoenolpyruvate carboxy kinase (PCK, Flpter 0.84), as well as anonymous cosmid clones RMC20C026 (Flpter 0.79), RMC20C001 (Flpter 0.825), and RMC20C030 (Flpter 0.85). In our pilot study, RMC20C001 was shown to define the region of maximum amplification (11). We also used one cosmid probe mapping to the proximal p-arm, RMC20C038 (FLpter 0.237), as a chromosome-specific reference probe.

FISH. Two-color FISH was performed using biotin-14-dATP-labeled 20q13-specific probes and digoxigenin-11-dUTP-labeled 20p reference probe as described (16). Tumor samples were postfixed in 4% paraformaldehyde-PBS for 5 min at 4°C prior to hybridization, dehydrated in 70, 85, and 100% ethanol, air dried, and incubated for 30 min at 80°C. Slides were denatured in a 70% formamide-2× SSC solution at 72–74°C for 3 min, followed by a proteinase K digestion (0.5 μg/ml). The hybridization mixture contained 18 ng of each of the labeled probes and 10 μg human placental DNA. After hybridization, the probes were detected immunochemically with avidin-FITC and antidigoxigenin rhodamine. Slides were counterstained with 0.2 μM DAPI in an antifade solution.

Fluorescence Microscopy and Scoring of Signals in Interphase Nuclei. A Nikon fluorescence microscope equipped with double band-bass filters (Chromatechnology, Brattleboro, VT) and a ×63 objective (numerical aperture 1.3) were used for simultaneous visualization of FITC and rhodamine signals. At least 50 nonoverlapping nuclei with intact morphology based on the DAPI counterstaining were scored to determine the number of test and reference probe hybridization signals. Leukocytes infiltrating the tumor were excluded from analysis. Control hybridizations to normal fibroblast interphase nuclei were done to ascertain that the probes recognized a single copy target and that the hybridization efficiencies of the test and reference probes were similar. The scoring results were expressed both as the actual copy number/cell (mean number of hybridization signals/cell) and as the mean level of amplification (mean of number of signals from the test probe relative to the number of reference probe signals).

CGH. Nine highly amplified and 22 tumors with no 20q13 amplification were analyzed by CGH as described elsewhere (6, 17, 18). Briefly, tumor DNA samples from the tumors were labeled with FITC-dUTP (DuPont, Boston, MA), and normal female DNA was labeled with Texas Red-dUTP (DuPont) using nick translation. Labeled DNAs (400 ng each) and 10 μg of unlabeled Cot-1 DNA (GIBCO-BRL, Gaithersburg, MD) were hybridized together to normal metaphase chromosomes. The hybridizations were evaluated using a digital image analysis system as described previously (6, 18). Green:red (tumor:normal) fluorescence ratio profiles were calculated for each chromosome from pter to qter to determine the copy number changes in the tumors.

DNA Flow Cytometry and Steroid Receptor Analyses. DNA flow cytometry was performed from frozen 200-μm sections as described previously (19). Analysis was carried out using an EPICS C flow cytometer (Coulter Electronics Inc., Hialeah, FL) and the MultiCycle program (Phoenix Flow Systems, San Diego, CA). A DNA index over 1.07 (in over 20% of cells) was used as a criterion for DNA aneuploidy. In DNA aneuploid histograms, the S-phase was analyzed only from the aneuploid clone. Cell cycle evaluation was successful in 86% (108/126) of the tumors.

ER and PR were detected immunohistochemically from cryostat sections as previously described (20). The staining results were semiquantitatively evaluated, and a histoscore greater than or equal to 100 was considered positive for both ER and PR (20).

Statistical Methods. Contingency tables were analyzed with the χ^2 test for trend. Association between the S-phase fraction (continuous variable) and 20q13 amplification was analyzed with the Kruskal-Wallis test. Analysis of disease-free survival was performed using the BMDP1L program and Mantel-Cox test, and Cox's proportional hazards model (BMDP2L program) was used in multivariate regression analysis (21). Association between the number of genetic changes seen by CGH and high-level 20q13 amplification were analyzed with the Mann-Whitney *U* test. *P* values were two-tailed.

Results

Amplification of 20q13 in Primary Breast Carcinomas by FISH. The previously defined (11) minimal region probe RMC20C001 was used in FISH analysis to assess the 20q13 amplification. Both the total number of signals in individual tumor cells and the mean level of amplification (mean copy number with the RMC20C001 probe relative to a 20p reference probe) were determined. In addition, the intratumor heterogeneity in signal copy numbers was also evaluated. Tumors were classified into three categories: no, low, and high level of

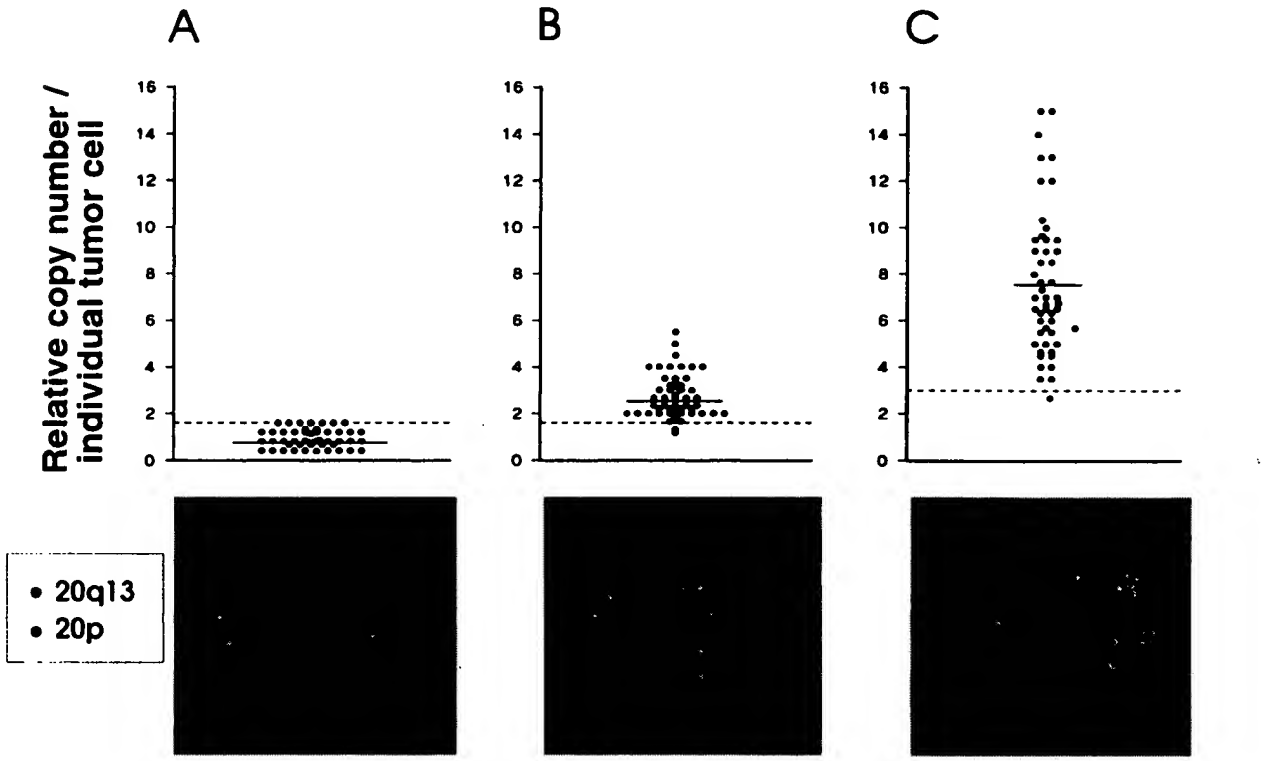

Fig. 1 Examples of two-color FISH analyses of breast cancer nuclei in three tumors with no (*A*), low-level (*B*), and high-level (*C*) 20q13 amplification. 20q13 probe signals are visualized in *green* and those of the 20p reference probe in *red* fluorescence. Nuclei were counterstained with DAPI (*blue*). The corresponding scattergrams indicate levels of amplification in individual tumor cells from these three tumors. *Bars*, average level of amplification. *Dashed lines*, cutoff for low-level amplification (1.5 in *A* and *B*) and high-level amplification (3.0 in *C*). *A*, a tumor with two signals with both 20q13 and 20p probes, suggesting disomy for chromosome 20 and no amplification. *B*, a slightly increased number of 20q13 signals as compared to 20p, suggesting low-level amplification. *C*, a highly increased number of 20q13 signals arranged in clusters, suggesting high-level 20q13 amplification. ×1000.

amplification. Tumors classified as not amplified showed <1.5 fold copy number of the RMC20C001 as compared to the p-arm control (Fig. 1*A*). Those classified as having low-level amplification had a 1.5–3-fold average level of amplification (Fig. 1*B*). Tumors showing >3-fold average level of amplification were classified as highly amplified (Fig. 1*C*). The highly amplified tumors often showed extensive intratumor heterogeneity in individual tumor cells containing up to 40 signals/cell. The intratumor heterogeneity is demonstrated in Fig. 1, where the distribution of 20q13 copies in individual cells is plotted for three tumors. In highly amplified tumors, the RMC20C001 probe signals were always arranged in clusters by FISH, which indicates location of the amplified DNA sequences in close proximity to one another, *e.g.*, in a tandem array (Fig. 1*C*). Low-level 20q13 amplification was found in 29 (22%) of the 132 primary tumors, whereas 9 (6.8%) cases showed high-level amplification. The overall prevalence of increased copy number in 20q13 was thus 29% (38/132).

Defining the Minimal Region of Amplification. The average copy number of four probes flanking RMC20C001 was determined in the nine highly amplified tumors. The flanking probes tested were melanocortin-3 receptor (MC3R, FLpter 0.81), phosphoenolpyruvate carboxykinase (PCK, 0.84), RMC20C026 (0.79), and RMC20C030 (0.85). The amplicon size and location varied slightly from one tumor to another, but RMC20C001 was the only probe consistently highly amplified in all nine cases (Fig. 2).

Association of 20q13 Amplification with Pathological and Biological Features. 20q13 amplification was associated with a high histological grade of the tumors ($P = 0.01$). This correlation was seen both in moderately and highly amplified tumors (Table 1). 20q13 amplification was also associated with aneuploidy by DNA flow cytometry ($P = 0.01$). The mean cell proliferation activity, measured as the percentage of cells in the S-phase fraction, increased ($P = 0.0085$) along with the level of amplification in the tumors. No association was found between 20q13 amplification and the age of the patient, primary tumor size, axillary nodal, or steroid hormone receptor status.

Relationship between 20q13 Amplification and Disease-free Survival. Disease-free survival of patients with high-level 20q13 amplification was significantly shorter than for patients with either no amplification or those with low-level amplification ($P = 0.04$, Fig. 3*A*). Disease-free survival of patients with tumors of low-level amplification did not differ significantly from that of patients with no amplification. Among the node-negative patients ($n = 79$), high-level 20q13

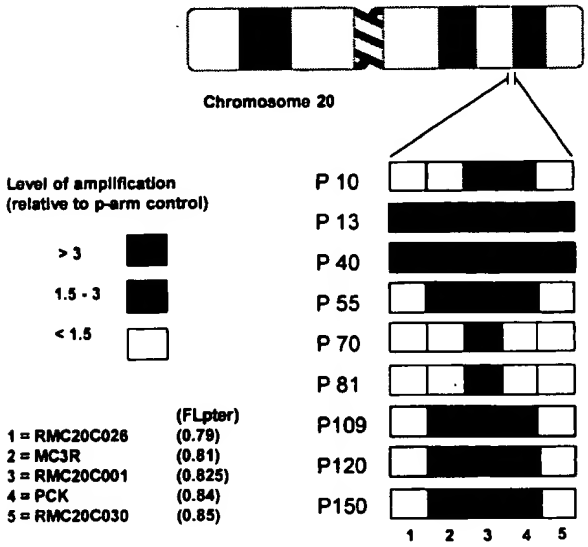

Fig. 2 Defining the minimal common region of amplification in nine primary breast tumors using two P1 probes to known genes and three cosmid probes to anonymous loci at 20q13. RMC20C001 is the only probe consistently highly amplified in all nine tumors.

amplification was a highly significant prognostic factor of short disease-free survival ($P = 0.002$; Fig. 3*B*). This prognostic effect persisted in Cox's multivariate regression analysis ($P = 0.026$) after adjustment for the prognostic value of tumor size, ER, PR, grade, ploidy, and S-phase fraction.

20q13 Amplification in Metastatic Breast Tumors. Two of 11 metastatic breast tumors had low-level and 1 had high-level 20q13 amplification. Thus, the overall prevalence (27%) of increased 20q13 copy number in metastatic tumors was similar to that observed in the primary tumors. Both primary and metastatic tumor specimens were available from one of the patients. This 29-year-old patient developed a pectoral muscle infiltrating metastasis 8 months after total mastectomy. The patient did not receive adjuvant or radiation therapy after mastectomy. The majority of tumor cells in the primary tumor showed low-level amplification, although individual tumor cells (<5% of total) contained 8–20 copies/cell by FISH (Fig. 4.). In contrast, all tumor cells from the metastasis showed high-level 20q13 amplification (12–50 copies/cell). The absolute copy number of the reference probe remained the same, suggesting that the higher level of amplification in the metastasis was not a result of an increased degree of aneuploidy (Fig. 4).

Correlation of 20q13 Amplification with Other Genetic Aberrations. According to CGH results, tumors with high-level 20q13 amplification showed a significantly higher number of genetic aberrations than those with no amplification ($P = 0.02$). Tumors with high-level amplification characteristically ($P = 0.012$) showed losses of many different chromosomes and chromosomal regions. In contrast, there were no differences in the total number of gains and amplifications of DNA sequences between the two patient groups (Table 2.). When the specific regions of involvement were studied, the most common aberrations in the nine tumors with high-level 20q13 amplification

Table 1. Clinicopathological correlations of amplification at chromosomal region 20q13 in 132 primary breast cancers

	20q13 Amplification status			
	No	Low level	High level	
Pathobiological feature	No. of patients	No. of patients	No. of patients	P^a
All primary tumors	94 (71%)	29 (22%)	9 (6.8%)	
Age of patients (yr)				
<50	17 (65%)	6 (23%)	3 (12%)	
≥50	77 (73%)	23 (22%)	6 (5.7%)	0.39
Tumor size (cm)				
<2	33 (79%)	7 (17%)	2 (4.8%)	
≥2	58 (67%)	22 (25%)	7 (8.0%)	0.16
Nodal status				
Negative	49 (67%)	19 (26%)	5 (6.8%)	
Positive	41 (75%)	10 (18%)	4 (7.3%)	0.41
Histological grade				
I–II	72 (76%)	18 (19%)	5 (5.3%)	
III	16 (52%)	11 (35%)	4 (13%)	0.01
ER status				
Negative	30 (67%)	10 (22%)	5 (11%)	
Positive	59 (72%)	19 (23%)	4 (4.9%)	0.42
PR status				
Negative	57 (69%)	20 (24%)	6 (7.2%)	
Positive	32 (74%)	8 (19%)	3 (7.0%)	0.53
DNA ploidy				
Diploid	45 (82%)	8 (14.5%)	2 (3.6%)	
Aneuploid	44 (62%)	20 (28%)	7 (10%)	0.01
S-phase fraction (%)				
Mean ± SD	9.9 ± 7.2	12.6 ± 6.7	19.0 ± 10.5	0.0085[b]

[a] χ^2 test for trend.
[b] Kruskal-Wallis test.

were: 8q+ (five cases), 11q− (five cases), 13q− (six cases), and 18q− (six cases). Only three of the nine tumors showed high-level amplifications (one in 11p and two in 8q) other than the 20q amplification.

Discussion

The present findings suggest that 20q13 amplification may be an important component of the genetic progression pathway of certain breast carcinomas. Specifically, the new findings of this study were: (*a*) High-level 20q13 amplification, detected in 7% of the tumors, was significantly associated with decreased disease-free survival in node-negative breast cancer patients, as well as with indirect indicators of high malignant potential, such as high grade and S-phase fraction. (*b*) Low-level amplification, which was much more common, was also associated with clinicopathological features of aggressive tumors, but showed no prognostic significance. (*c*) The level of amplification of RMC20C001 is higher than amplification of nearby candidate genes and loci, indicating that a novel oncogene is located in the vicinity of RMC20C001.

High-level 20q13 amplification was defined by the presence of more than a 3-fold higher copy number of the 20q13 region than of the p-arm reference region and by the finding of clusters of 20q13 probe signals in the tumor interphase nuclei. The prevalence of high-level 20q13 amplification is somewhat lower than the amplification frequencies reported for some of the other breast cancer oncogenes, such as *erb-B2* and *cyclin D*

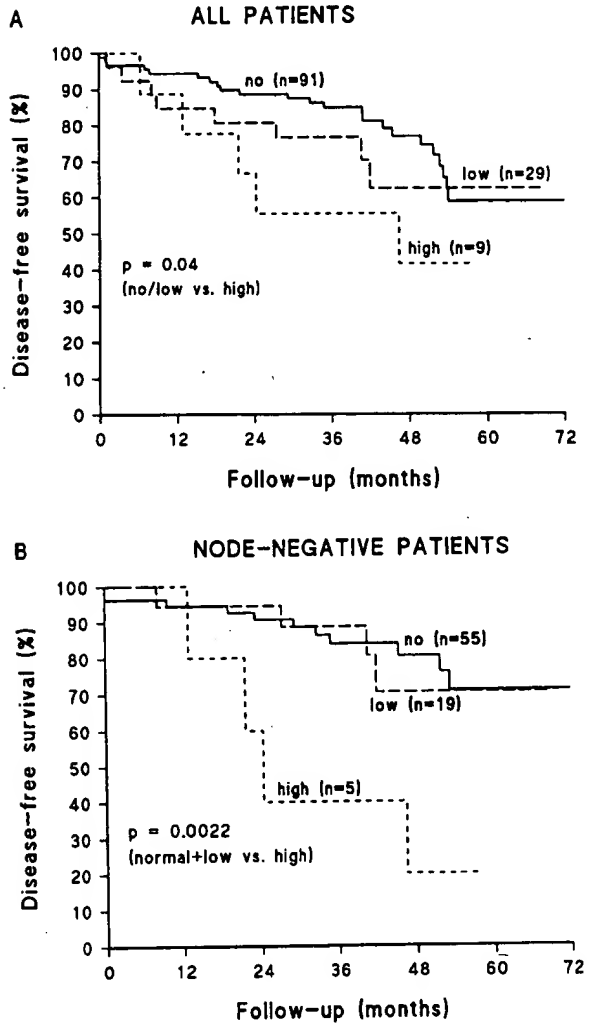

Fig. 3 A, disease-free survival of 129 breast cancer patients according to the level of 20q13 amplification. Patients with tumors having high-level 20q13 amplification have a shorter disease-free survival ($P = 0.04$, Mantel-Cox test) compared to those having no amplification or low-level amplification. *B*, the same disease-free survival difference is observed more clearly in the subgroup of 79 axillary node-negative patients ($P = 0.0022$, Mantel-Cox test).

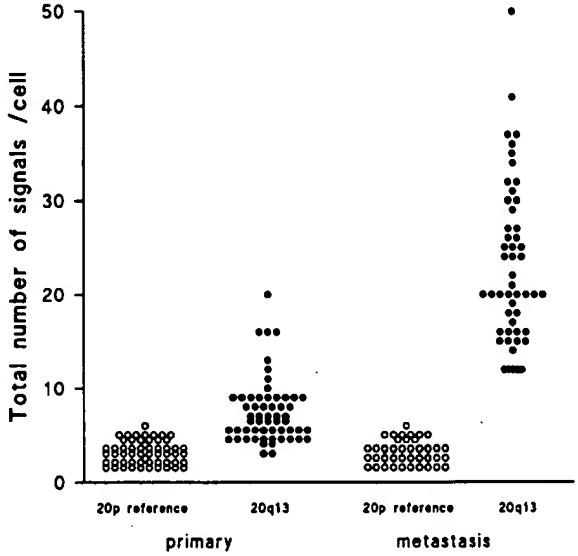

Fig. 4 Comparison of 20q13 amplification detected by FISH in a primary breast carcinoma and its metastasis from a 29-year-old patient. A low-level amplification of 20q13 was found in the primary tumor. The metastasis, which appeared 8 months after mastectomy, shows a high-level amplification of the chromosome 20q13 region. The overall copy number of chromosome 20 (20p reference probe) remained unchanged. *Each data point*, gene copy numbers in individual tumor cells analyzed from the two specimens.

Table 2. Comparison of the overall number of genetic aberrations seen by CGH in breast carcinomas with no 20q13 amplification and in those with high-level amplification at this locus by FISH

	Median no. of aberrations/tumor (range)		
CGH findings	No 20q13 amplification ($n = 22$)	High-level 20q13 amplification ($n = 9$)	P^a
Gains and amplifications	2 (0–6)	3 (1–7)	0.14
Losses	2 (0–6)	8 (0–11)	0.012
Total	4.5 (0–11)	13 (1–18)	0.02

[a] Mann-Whitney *U* test.

(4, 5). However, similar to what has been previously found with these other oncogenes (2, 4), high-level 20q13 amplification was more common in tumors with high grade or high S-phase fraction and in cases with poor prognosis. Although a relatively small number of patients was analyzed, our results suggest that 20q13 amplification may be a significant and perhaps independent prognostic factor in node-negative breast cancer. Moreover, based on these survival correlations, the currently unknown, putative oncogene amplified in this locus may confer an aggressive phenotype. Thus, cloning of this gene is an important goal. Based on the association of amplification with highly proliferative tumors, one could hypothesize a role for this gene in the growth regulation of the cell.

Because of the association of 20q13 amplification with DNA aneuploidy, one cannot, however, exclude the possibility that this amplification is reflecting the fact that some tumors are genetically unstable. Indeed, CGH analyses showed that tumors with high-level 20q13 amplification had a significantly higher total number of genetic aberrations than those without amplification. This difference was only attributable to a higher number of chromosome losses in the 20q13 positive tumors, while DNA gains and amplifications in the two groups were equally abundant. We have found by CGH that tumors with *erb-B2* oncogene amplification also have a 2-fold higher total number of genetic aberrations than tumors with no *erb-B2* amplification.[5] Since

[5] J. Isola and F. Waldman, unpublished data.

genetic instability is a prerequisite for DNA amplification, amplification of any oncogene is likely to be most common in genetically advanced tumors. Interestingly, breast cancers with high-level 20q13 amplification showed a characteristic overall pattern of genetic aberrations. Losses of chromosomal regions 11q13-qter and 18q were the most common genetic changes in these tumors, whereas only 6–13% of other breast carcinomas studied by CGH have been reported to contain these aberrations (22).[6] The biological significance of 20q13 amplification (as well as that of any other gene amplification) may be influenced by a large number of other genetic aberrations that these genetically unstable tumors contain. Thus, very large studies are now needed to address the independent prognostic significance of each of the individual genetic aberrations.

The role of the low-level 20q13 amplification as a significant event in tumor progression appears to be less clear. Low-level amplification was defined as a 1.5–3-fold increased average copy number of the 20q13 probe relative to the p-arm control. In addition, these tumors lacked individual tumor cells with very high copy numbers and showed a scattered, not clustered, appearance of the signals. Accurate distinction between high- and low-level 20q13 amplification can only be reliably done by FISH, whereas Southern and slot blot analyses are likely to be able to detect only high-level amplifications. This distinction is important, because only the highly amplified tumors were associated with adverse clinical outcome. Tumors with low-level 20q13 amplification were characterized by many clinicopathological features that were in between those found for tumors with no amplification and those with high-level amplification. Low-level amplification may precede the development of high-level amplification as shown by, *e.g.*, the development of drug resistance-gene amplification *in vitro* (23). In one of our patients, a local metastasis contained a much higher level of 20q13 amplification than the primary tumor operated on 8 months earlier.

In our previous publication, we reported a 1.5-megabase critical region defined by RMC20C001 probe and exclusion of candidate genes in breast cancer cell lines and in a limited number of primary breast tumors. Results of the present study confirm these findings by showing conclusively in a larger set of primary tumors that the critical region of amplification is indeed defined by this probe.

The present data suggest that the high-level 20q13 amplification may be a significant step in the progression of certain breast tumors to a more malignant phenotype. The prognostic implications of 20q13 amplification are promising and warrant further studies. Approximate location of the minimal region of amplification at 20q13 has now been defined.

[6] M. Tirkkonen, personal communication.

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